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Toll-like receptor 6 mediated inflammatory and functional responses of zinc oxide nanoparticles primed macrophages

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Summary

Macrophages are among the most sensitive immune cells because of their phagocytic activity and are prone to become dysfunctional or not able to perform properly if nanoparticle load increases. We have previously reported that zinc oxide nanoparticles (ZNPs) induce inflammatory responses in macrophages that contribute to their death. Recognition of ZNPs by pattern recognition receptors such as toll-like receptors (TLRs) might be a factor in the initiation of these responses in macrophages. Therefore, in this study we explored the role played by TLR6 and mitogen-activated protein kinase (MAPKs) pathways in the inflammatory responses of macrophages during ZNPs exposure. ZNPs-activated macrophages showed enhanced expression of activation and maturation markers (CD1d, MHC-II, CD86 and CD71). Among various TLRs screened, TLR6 emerged as the most potent activator for ZNPs-induced inflammatory responses. Downstream signalling proteins myeloid differentiation 88, interleukin-1 receptor associated kinase and tumour necrosis factor receptor-associated factor were also enhanced. On inhibiting MAPKs pathways individually, the inflammatory responses such as interleukin-1β, interleukin-6, tumour necrosis factor-α, cyclooxygenase-2 and inducible nitric oxide synthase were suppressed. TLR6 silencing significantly inhibited the pro-inflammatory cytokine levels, reactive nitrogen species generation and inducible nitric oxide synthase expression. Also, inhibition of MAPKs in the absence of TLR6 signalling validated the link between TLR6 and MAPKs in ZNPs-induced inflammatory responses. TLR6 was found to be co-localized with autophagosomes. Macrophages lacking TLR6 inhibited the autophagosome marker protein-microtubule-associated protein1 light chain 3-isoform II formation and phagocytosis. These results demonstrate that inflammatory responses caused by ZNPs-activated macrophages strongly depend on TLR6-mediated MAPK signalling.

Keywords: inflammation; macrophages; nanoparticles; toll-like receptors.

Introduction

Zinc oxide nanoparticles (ZNPs) are among the most commonly used nanomaterials, with a wide range of applications in industrial and consumer products. Recently, ZNPs have been shown to specifically target and kill cancer cells and can possibly be developed as an alternative anticancer therapeutic agent.[1,2] They have also been reported to have the potential to activate dendritic cells and stimulate the release of pro-inflammatory cytokines.[3] In particular, workers in the manufacturing of ZNPs can be subjected to low-level chronic exposure to ZNPs via different routes, namely inhalation, ingestion or dermal route. Ryman-Rasmussen *et al.*,[4] have shown that sub-micrometre particles can penetrate even through the outer layers of the skin.

In the present study, macrophages were chosen as an *in vitro* model to study the exposure to ZNPs because they are the primary scavenger cells of the body and form the first line of defence in the immune response to foreign materials.[5] They are located in the skin, lungs and body cavities where they catch and engulf foreign material

to further process them as antigenic molecules for other immune cells to react to. After engulfment, macrophages usually mature to become active antigen-presenting cells (APCs) that express maturation markers [cluster of differentiation (CD11b)] and various activation markers (CD71, CD80, CD86) on their surfaces and present antigens on their MHC and CD1d molecules.[6–9] It has been revealed that the innate immune system can recognize foreign invaders through Toll-like receptors (TLRs) expressed on the cell surface of immune cells. The TLRs are pattern-recognition receptors that have a unique and essential function in animal immunity. Recent studies have shown that TLRs have a crucial role in the recognition of the molecular signature of microbial infection, inducing different signalling pathways. The expression of TLRs in murine peritoneal macrophages may be an important aspect for their activation.[10] Activated APCs produce cytokines and chemokines that attract responder cells such as neutrophils and other inflammatory cells to the site of the inflammation.[9]

When nanoparticles enter the systemic circulation, they encounter a complex web of immune cells and plasma proteins. The recognition of nanoparticles as non-self by the immune cells may lead to the generation of reactive oxygen species, reactive nitrogen species and altered cytokine levels. Abnormal levels of pro-inflammatory cytokines like tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-17 and interferon-γ have been reported in rheumatoid arthritis, active psoriasis and allergic asthma.[11–13] On inflammatory stimulation, cyclo-oxygenase-2 (COX-2) is induced, which is the most important source of prostanoid formation during inflammation.[14] Reactive nitrogen species generation is another major product of inflammation and its production is controlled by nitric oxide synthase (NOS).

Mitogen-activated protein kinases (MAPKs) are recognized as one of the most versatile signalling kinases that link upstream signalling events in the regulation of various inflammation-related gene expressions in multiple cell types in response to a wide range of stimuli.[15,16] Our previous studies showed that ZNPs have the immunomodulatory potential to alter the pro-inflammatory cytokines by activating MAPKs [phospho-extracellular signal-regulated kinase (p-ERK), phospho-c-Jun N-terminal kinase (p-JNK) and phospho-protein 38 (p-p38)] after internalization into the macrophages.[17] ZNPs also induced inflammatory responses in different organs such as intestine, lung and spleen by activating macrophages through activation of MHC-II and CD11b.[18,19] It is known that recognition of nanoparticles by TLRs is necessary for the activation of host defence mechanisms.[20]

Collectively these reports influenced us to explore whether TLRs are required for recognition of ZNPs and are necessary to initiate immune responses. There might be links among ZNPs exposure, TLRs activation, MAPKs signalling and inflammation that might be of particular importance. Therefore, the objectives of this study were to determine the potential pattern recognition receptors for ZNPs and investigate the associations between TLRs and MAPKs involved in the inflammatory responses generated by ZNPs exposure.

Materials and methods

Macrophage culture

Inbred strains of female BALB/c mice (8–10 weeks old) were killed according to the guidelines for the care and use of laboratory animals of the Animal Ethics Committee of the CSIR-Indian Institute of Toxicology Research, Lucknow, India. This study was carried out after the approval of the Institutional Ethics Committee (approval no. IITR/IAEC/47/11). Peritoneal exudate cells were collected from the peritoneal cavity of mice by injecting chilled RPMI-1640 medium and added to 96-well cell culture flat bottom plates. After 3 hr of incubation in a CO_2 incubator (5% CO_2) at 37°, the non-adherent cells were removed by vigorous washing (three times) with warm RPMI-1640 medium. Furthermore, adhered cells were incubated overnight in RPMI-1640 medium supplemented with heat-inactivated 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml) at 37° in the humid air containing 5% CO_2 to form macrophage monolayers. More than 95% of the adherent cell populations were macrophages, as determined by morphology and non-specific esterase staining.

Cell surface molecules

Activation state of macrophages was decided by analysing different surface markers for cell maturation, activation and antigen presentation. These markers were studied after 24 hr of exposure to 2·5 μg/ml of ZNPs (dose selected from a previous study).[17] Macrophages were removed by gentle scraping followed by pipetting. After two washes with ice-cold PBS, cells were stained with Alexa Fluor 488-conjugated CD11b, FITC-conjugated CD1d, FITC-conjugated CD86, FITC-conjugated MHC-II and phycoerythrin-conjugated CD71 (BD Bioscience, Frankllin Lakes, NJ). After a 45-min incubation period, samples were washed with PBS and analysed by flow cytometry.

Effect of TLR6 on inflammatory responses

The influence of TLR6 on inflammatory responses was seen by blocking TLR6 and then the expression of IL-1β, IL-6, TNF-α and inducible NOS (iNOS) and COX-2 were assessed. Mouse TLR6–small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNA was transfected into macrophages

by using the transfection reagent (Santa Cruz Biotechnology) and further macrophages were treated with 2·5 μg/ml of ZNPs for 24 hr.

Real-time PCR

For analysing the effect of MAPK signalling and TLR6 on inflammatory cytokine production, macrophages were pre-treated with MAPK inhibitors (10 μM PD98059 for ERK1/2, 10 μM SB203580 for JNK1/2 and 10 μM SP600125 for p38 for 1 hr) followed by 2·5 μg/ml of ZNPs for 24 hr and in another experiment macrophages were transfected with TLR6-siRNA before ZNPs (2·5 μg/ml of ZNPs for 24 hr) exposure. RT-PCR analysis of different TLRs (*TLR 1-9*), *iNOS* and cytokines (*IL-1β*, *IL-6* and *TNF-α*) in macrophages were carried out using gene-specific primers listed in Table 1. Total RNA from cells was isolated with RNAzol®RT (Molecular Research Center, Inc. Cincinnati, OH). cDNA from different groups were prepared by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturers' instructions. Quantitative real time-PCR was carried out using a SYBER Green PCR master mix (Thermo Scientific, Waltham, MA) according to the manufacturers' instructions. Amplification of house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was taken as an endogenous control. Normalized values have been used for plotting the bar graphs.

ELISA for assessment of cytokines

In the first set of experiments, murine peritoneal macrophage monolayers were treated with 2·5 μg/ml of ZNPs alone, pre-treated with p-ERK, p-p38 and p-JNK inhibitors (10 μM PD98059 for ERK1/2, 10 μM SB203580 for JNK1/2 and 10 μM SP600125 for p38 for 1 hr) followed by 2·5 μg/ml of ZNPs for 24 hr of incubation. In another set of experiments, the macrophage monolayers were TLR6–siRNA transfected followed by 2·5 μg/ml of ZNPs for 24 hr. Supernatants were collected and IL-1*β*, IL-6 and TNF-α were measured using an ELISA kit (BD Biosciences, San Diego, CA) according to the manufacturers' protocol.

Western blot analyses

For Western blot analysis, TLR2, TLR6, myeloid differentiation 88 (MyD88), IL-1 receptor associated kinase (IRAK) and TNF receptor-associated factor (TRAF) (Santa Cruz Biotechnology) were analysed in the cell lysates of untreated and ZNPs-treated macrophages after 0·5, 3, 6, 12 and 24 hr of incubation with 2·5 μg/ml of ZNPs.

COX-2 and iNOS (Calbiochem, Billerica, MA) were analysed in the cell lysates of untreated and treated groups of 2·5 μg/ml of ZNPs alone for 24 hr and different inhibitor pre-treated groups (PD98059 for ERK1/2, SB203580 for JNK1/2 and SP600125 for p38) which were followed by the exposure of 2·5 μg/ml of ZNPs for 24 hr.

Effect of TLR6 on MAPKs and TLR signalling, the protein expressions were analysed in cell lysates of control, TLR6-silenced control, treated and TLR6-silenced ZNPs-treated (2·5 μg/ml of ZNPs for 24 hr of incubation) macrophages. In these groups, p-JNK (BD Biosciences), p-ERK1/2, p-p38, cJun, nuclear factor-κB, phospho-activating transcription factor 2 (p-ATF2; Santa Cruz Biotechnology), TLR2, MyD88, IRAK and TRAF were analysed.

Proteins were resolved on 10% SDS polyacrylamide gel and transferred to PVDF membranes. The blotted membranes were blocked with 5% BSA in PBS containing 0·1% Tween 20 (PBS-T) and incubated with the desired antibodies at the dilutions mentioned by the manufacturer, followed by incubation with horseradish peroxidase-conjugated

Table 1. Primer sequences used for quantitative PCR

Gene	Forward primer	Reverse primer
TLR-1	5′- CTGAGGGTCCTGATAATGTCCT-3′	5′- TCCAGCTCTGTGTTGAATTTGA -3′
TLR-2	5′- GGGGCTTCACTTCTCTGCTT-3′	5′- AGCATCCTCTGAGATTTGACG-3′
TLR-3	5′- GATACAGGGATTGCACCCATA-3′	5′- TCCCCCAAAGGAGTACATTAGA-3′
TLR-4	5′-GGACTCTGATCATGGCACTG-3′	5′-CTGATCCATGCATTGGTAGGT-3′
TLR-5	5′-CTGGAGCCGAGTGAGGTC-3′	5′-CGGCAAGCATTGTTCTCC-3′
TLR-6	5′-GGTACCGTCAGTGCTGGAA-3′	5′-GGGTTTTCTGTCTTGGCTCA-3′
TLR-7	5′-GATCCTGGCCTATCTCTGACTC-3′	5′-CGTGTCCACATCGAAAACAC-3′
TLR-8	5′-CAAACGTTTTACCTTCCTTTGTCT-3′	5′-ATGGAAGATGGCACTGGTTC-3′
TLR-9	5′-GAGAATCCTCCATCTCCCAAC-3′	5′-CCAGAGTCTCAGCCAGCAC-3′
IL-1β	5′-AAGAGCTTCAGGCAGGCAGTATCA-3′	5′-TGCAGCTGTCTAATGGGAACGTCA-3′
IL-6	5′- ATCCAGTTGCCTTCTTGGGACTGA-3′	5′- TAAGCCTCCGACTTGTGAAGTGGT-3′
TNF-α	5′-TCTCATGCACCACCATCAAGGACT3′	5′-ACCACTCTCCCTTTGCAGAACTCA-3′
iNOS	5′- CTGCTGGTGGTGACAAGCACATTT-3′	5′- ATGTCATGAGCAAAGGCGCAGAAC-3′
GAPDH	5′-TTCACCACCATGGAGAAGGC-3′	5′-GGCATGGACTGTGGTCATGA-3′

secondary antibody (Sigma, St Louis, MO). Bound secondary antibody was detected by enhanced chemiluminescence using Amersham ECL Western blotting detection reagents as described in the manufacturers' protocol (Amersham, Fairfield, CT). All the blots were stripped and reprobed with β-actin to ensure equal loading of protein.

The density of each band was estimated by the GENETOOLS software (Syngene, Bangalore, India). The β-actin was taken as an endogenous control and its respective densitometry values were used for normalizing different protein expressions. Normalized values were used to calculate relative fold change with respect to control of each blot and derived values have been indicated above the blots.

Measurement of reactive nitrogen species generation

In all, 4×10^5 cells/well were seeded into 96-well cell culture plates. Cells were incubated with 2·5 μg/ml of ZNPs for 24 hr. In other sets, cells were transfected with TLR6–siRNA followed by treatment with ZNPs. Serial dilutions of nitrite standard solutions were made with nitrite concentrations ranging from 0·1 to 20 μM. Thereafter, in culture supernatants, reactive nitrogen species were measured using Griess reagent. The absorbance was taken at 540 nm in a plate reader (BioTek, Winooski, VT). To assess whether the autofluorescence or absorbance of ZNPs interferes with the Griess reagent, a set of experiments without cells were conducted in parallel.

Immunofluorescence staining

To determine the localization of TLR6 within the phagosomes, the untreated and ZNPs-treated (2·5 μg/ml dose for 24 hr) macrophages were washed and fixed in 4% paraformaldehyde. These fixed macrophages were blocked with 3% BSA for 0·5 hr followed by anti-TLR6 antibody overnight at 4°. After gentle washing with PBS-T, cells were probed with Alexa Flour 546 conjugated anti-goat antibody and FITC-tagged microtubule-associated protein1 light chain 3-isoform (MAP1-LC3) antibody (Cyto-ID® autophagy detection kit; Enzo Life Sciences, Lausen, Switzerland) for 2 hr to detect co-localization of TLR6 within autophagosomes. The nucleus was stained with Hoechst 33342 stain. After the completion of staining, cells were washed twice with 1× assay buffer provided in the kit. Slides were observed and photographed under a confocal microscope (Leica Microsystems, Wetzlar, Germany). Respective differential interference contrast images were also captured.

Phagocytic activity in TLR6-silenced macrophages

Phagocytosis is one of the characteristic properties of activated macrophages. Therefore, the effect of TLR6 on the phagocytic property that is linked to the phagosome formation was also determined. We used carboxylate-modified polystyrene latex beads (Sigma) that were opsonized by mixing with fetal bovine serum for 0·5 hr at 37°. TLR6–siRNA-transfected macrophages were treated with 2·5 μg/ml of ZNPs for 24 hr. After incubation, the ZNPs were removed by washing with 1× PBS followed by the addition of beads in a ratio of 10 : 1 (beads : cell). After incubation for 0·5 hr, samples were washed three times with 1× PBS and then wells were observed and photographed under an inverted light microscope (Olympus IX51; Olympus Optical Co. Ltd. Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± SE. The results were analysed using one-way analysis of variance. All the statistical analyses were performed using PRISM version 5, Graph Pad Software Inc. (San Diego, CA). A value of $P < 0{\cdot}05$ was considered as statistically significant.

Results

Exposure to ZNPs induces changes in the cell-surface molecules of macrophages

To evaluate the extent of the activation state required for antigen presentation, we studied whether the ZNPs enhance cell maturation marker (CD11b), antigen presentation (MHC-II, CD1d), activation marker (CD71) and co-stimulation activity (CD86) of macrophages. In macrophages, ZNPs enhanced the expression of CD11b, MHC-II and CD1d after exposure (Fig. 1). CD11b, CD1d and MHC-II are prominently expressed on cells involved in antigen presentation, which suggests their role in the activation of immunity.

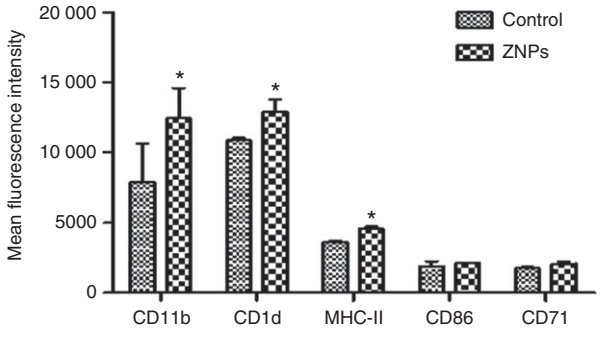

Figure 1. Zinc oxide nanoparticles (ZNPs) induced antigen presentation and activation markers in macrophages. Treatment with 2·5 μg/ml of ZNPs for 24 hr showed enhancement of antigen presentation, maturation and activation markers in macrophages. The data represent mean ± SE of three sets (*$P < 0{\cdot}05$, significant with respect to control).

Effect on TLRs and downstream signalling

For macrophages, TLRs serve as a major parameter for their activation. The mRNA expression of TLR1, TLR2, TLR4 and TLR6 in ZNPs-treated macrophages was evaluated and presented as fold change. The increase in expression of TLR1, TLR2 and TLR4 was almost eight-fold, nine-fold and seven-fold, respectively. TLR6 had the highest increase in expression, i.e. up to 12-fold. The mRNA expression of TLR3, TLR5, TLR7 and TLR8 was less compared with that of the TLRs described above (four-fold, six-fold, three-fold and 3·6-fold, respectively) while the expression of TLR9 was suppressed 0·73-fold (Fig. 2a). As a result, only the protein levels of TLR2 and TLR6 were investigated further, because their mRNA expressions were highest and also it is known that TLR6 forms a heterodimer with TLR2 to mediate its effects. We found that the protein levels of TLR6 were much higher than of TLR2, which showed the significant role of TLR6 in the ZNPs-induced responses (Fig. 2b). Further, ZNPs enhanced the downstream signalling molecules of TLRs such as MyD88 and TRAF (increased expression started from 3 hr and remained up to 24 hr); and IRAK (increased expression at 12 hr).

Effect of MAPKs inhibitors on the ZNPs-stimulated IL-1β release

To characterize the role of MAPKs in the ZNPs-induced secretion of the inflammatory cytokine IL-1β, macrophages were pre-treated with specific inhibitors of MAPKs intracellular signalling routes. Figure 3(a) shows that the mRNA expression of IL-1β (four-fold increase) was significantly inhibited in the presence of ERK inhibitor by 99%. The JNK and p38 inhibitors significantly decreased the amount of IL-1β induced by ZNPs by 98% and 99%, respectively.

The ERK and p38 inhibitors significantly inhibited secretion by the macrophages of IL-1β by up to 85%, whereas the JNK inhibitor decreased the release of IL-1β by up to 76% by the macrophages (Fig. 3b). These results collectively showed that IL-1β secretion induced by ZNPs was influenced by ERK and p38 pathways.

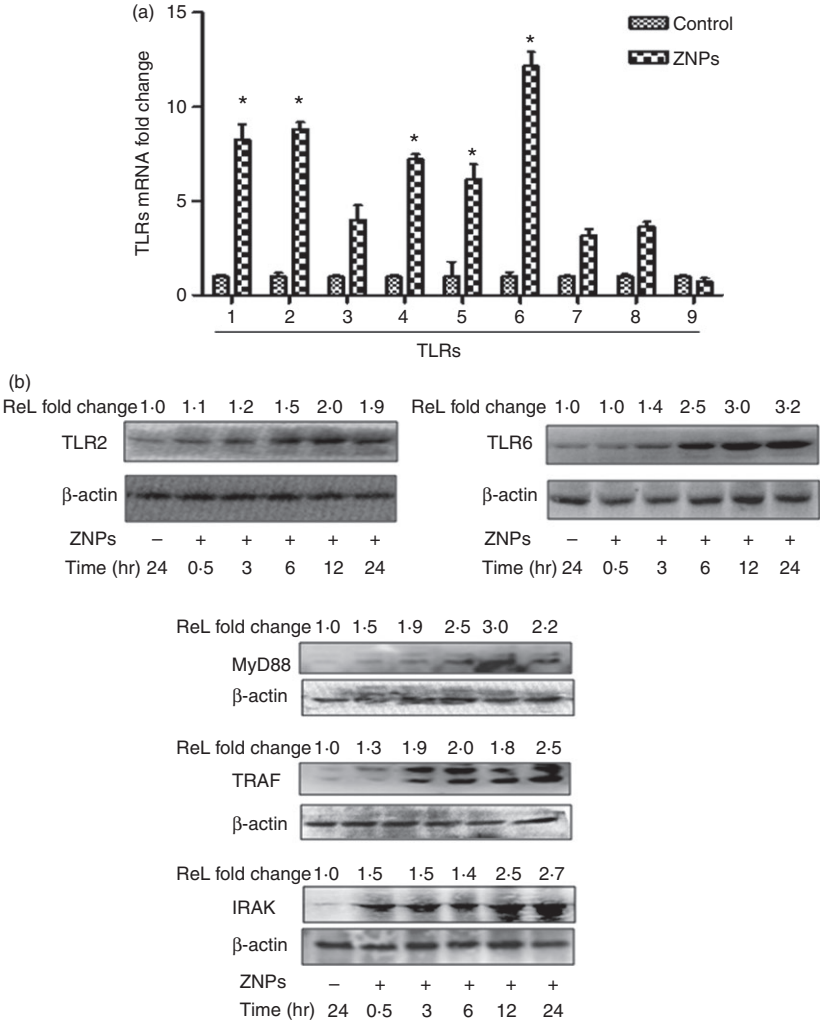

Figure 2. Identification of Toll-like receptors (TLRs) for the recognition of zinc oxide nanoparticles (ZNPs) and their downstream signalling molecules. Recognition of ZNPs by different TLRs, as quantified by mRNA expression, by RT-PCR after treatment of macrophages with 2·5 μg/ml ZNPs for 24 hr. Data represents mean ± SE, (n = 3); *P < 0·05 when compared with respective control. Representative blots showed the expression of TLR2, TLR6, myeloid differentiation 88 (MyD88), IL-1 receptor associated kinase (IRAK) and tumour necrosis factor receptor-associated factor (TRAF) on ZNPs treatment for 0·5, 3, 6, 12 and 24 hr. Relative fold change in protein levels compared with control was denoted above the blots.

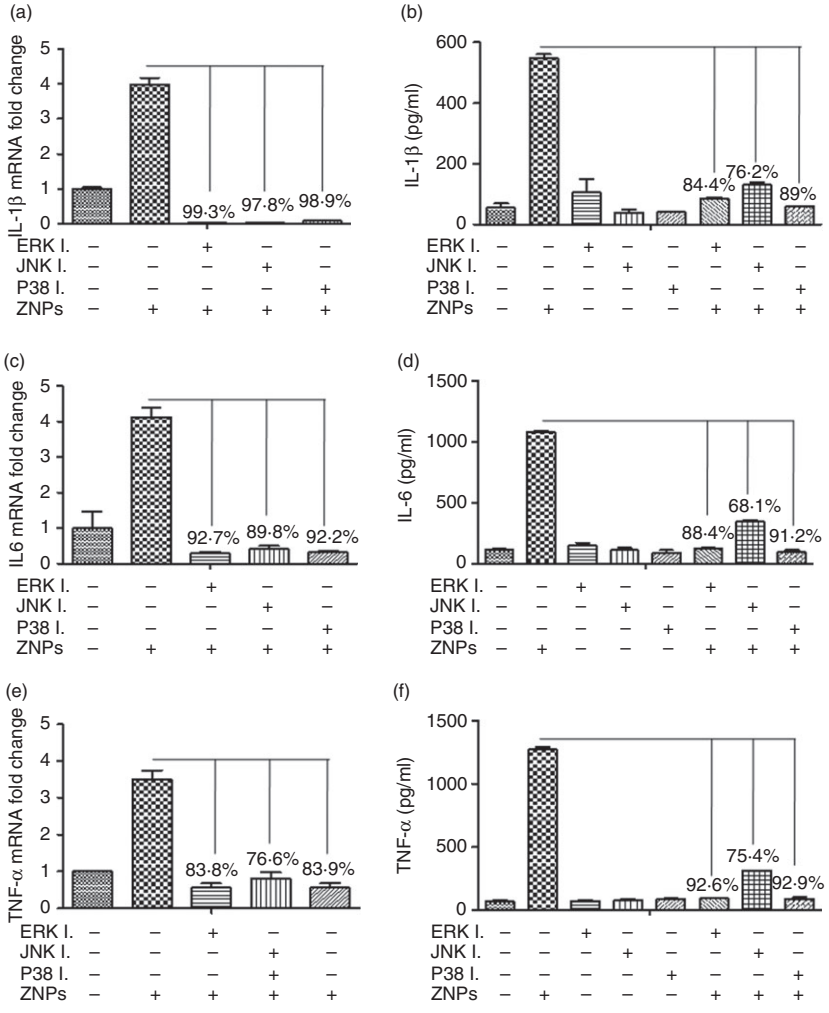

Figure 3. Effect of mitogen-activated protein kinase (MAPK) inhibitors on the zinc oxide nanoparticles (ZNPs) -stimulated pro-inflammatory cytokines. Messenger RNA and protein expression of (a, b) interleukin-1β (IL-1β), (c, d) IL-6 and (e, f) tumour necrosis factor-α (TNF-α) upon ZNPs alone and pre-treatment of inhibitors for extracellular signal regulated kinase (ERK), c-Jun-terminal kinase (JNK) and p38 followed by ZNPs exposure for 24 hr in macrophages was measured by RT-PCR and ELISA after treatment of macrophages with 2·5 μg/ml ZNPs for 24 hr. Data represents mean ± SE, (n = 3).

Effect of MAPKs inhibitors on the ZNPs-stimulated IL-6 release

Macrophages secreted large amounts of IL-6. We therefore further explored the effect of specific inhibitors of several intracellular signalling routes on ZNPs-induced IL-6 secretion (four-fold increase) triggered through different pattern recognition receptors. Figure 3(c) shows that the IL-6 mRNA was significantly decreased in the presence of all inhibitors assayed. In this case, the inhibition of ERK, JNK and p38 produced the most significant reduction in the IL-6 secretion induced by ZNPs (93, 90 and 92%, respectively).

The secretion of IL-6 by the macrophages was significantly inhibited by the p38 inhibitor, by 91%, while the ERK and JNK inhibitors decreased the release of IL-6 by up to 88 and 68%, respectively (Fig. 3d). The mRNA and protein quantification showed similar inhibition by ERK and p38 pathways for IL-6 secretion induced by ZNPs whereas the JNK pathway did not affect final protein levels.

Effect of MAPKs inhibitors on the ZNPs-stimulated TNF-α release

Tumour necrosis factor-α is a potent inflammatory cytokine involved in acute and chronic inflammatory diseases. For this reason, we next sought the effect of MAPKs inhibitors on the ZNPs-stimulated TNF-α release from macrophages. As can be observed in Fig. 3(e), secretion of TNF-α was decreased in the presence of all the inhibitors assayed. Stimulation of macrophages with ZNPs induced a significant strong increase in the secretion of TNF-α (3·4-fold increase), which was decreased by inhibitors of ERK and p38 (84% inhibition, by both inhibitors). In contrast, inhibition of JNK achieved a significant reduction in ZNPs-induced TNF-α secretion (77%) compared with ERK and p38.

Secretion of TNF-α by the macrophages was significantly inhibited by ERK and p38 inhibitors by nearly 93% while the JNK inhibitor decreased the release of TNF-α up to 75% (Fig. 3f). The mRNA and protein levels of TNF-α were similar for all three pathways concerned.

Here, TNF-α release on ZNPs exposure was influenced by ERK and p38 pathways.

Effect of MAPKs inhibitors on the ZNPs-stimulated COX-2 and iNOS expression

As shown in Fig. 4(a), the production of iNOS mRNA and protein was significantly down-regulated in ZNPs-treated macrophages that were pre-treated with inhibitors such as PD98059, SB202190 and SP600125. Pre-treatment of macrophages with PD98059, SB202190 and SP600125 inhibited very strongly the expression of iNOS but not COX-2 (Fig. 4b).

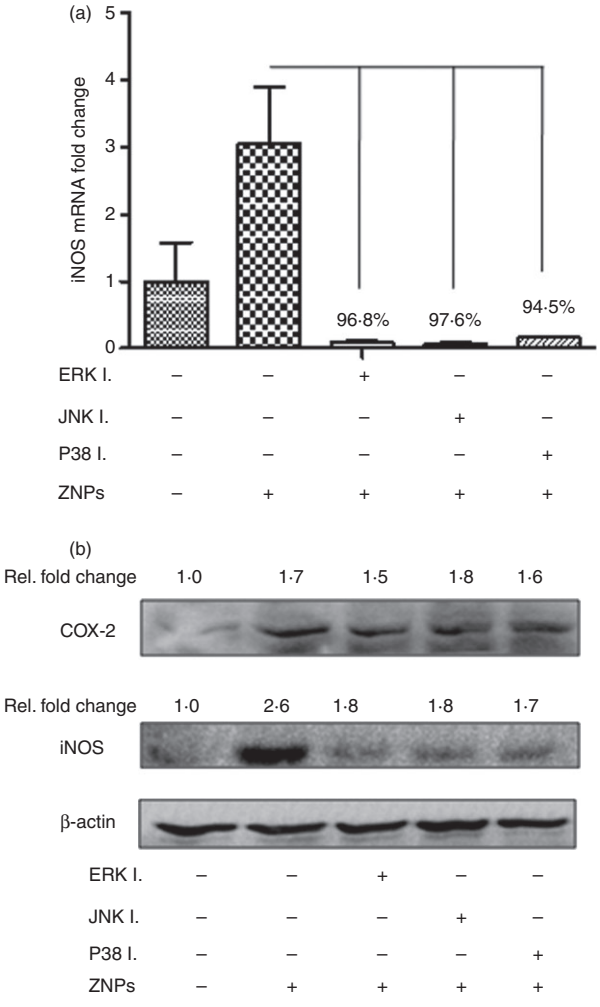

Figure 4. Involvement of mitogen-activated protein kinases (MAPKs) in zinc oxide nanoparticles (ZNPs) in cyclo-oxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) activation. (a) Messenger RNA expression of iNOS; (b) representative immunoblots of COX-2 and iNOS upon pre-treatment of inhibitors for extracellular signal regulated kinase (ERK), c-Jun-terminal kinase (JNK) and p38 followed by ZNPs exposure for 24 hr in macrophages. Blots were reprobed with anti-β-actin to ensure equal loading and relative fold change in protein levels compared with control was denoted above the blots. Data represents mean ± SE, (n = 3).

Effect of TLR6 on ZNPs-stimulated COX-2, iNOS, reactive nitrogen species and inflammatory cytokines

The role of TLR6 was assessed by blocking it with its siRNA; expression of IL-1β, IL-6 and TNF-α mRNA was significantly suppressed by 90, 81 and 70%, respectively, as shown in Fig. 5(a, c and e). The protein levels of IL-1β, IL-6 and TNF-α were also decreased, by 81, 86 and 89% respectively, showing similar pattern to the mRNA (Fig. 5b, d and f). Significant enhancement of reactive nitrogen species was observed after 24 hr of ZNPs exposure that was inhibited significantly, by 83%, on silencing TLR6 (Fig. 5g). The mRNA expression of iNOS was inhibited by 82.5% (Fig. 5h) and protein expression of COX-2 and iNOS were significantly inhibited compared with controls (Fig. 5i).

Linkage between TLR6 and MAPKs during ZNPs exposure

On silencing TLR6 signalling by TLR6–siRNA transfection, the mRNA and protein levels of TLR2 were suppressed, showing the direct effect of TLR6 on TLR2 and inhibiting downstream signalling proteins of TLRs such as MyD88, IRAK and TRAF (Fig. 6a). To clarify further the impact of TLR6 on MAPKs, their protein levels were analysed and the expression of p-ERK1/2 and p-p38 were found to be suppressed in the TLR6–siRNA + ZNPs group compared with ZNPs alone (Fig. 6b). In line with this finding, the downstream transcription factors such as nuclear factor-κB and ATF2 were also decreased whereas cJun was not affected at all (Fig. 6c).

Localization of TLR6 in autophagosomes

Here, we examined the localization of TLR6 in the phagosomes that were induced by ZNPs exposure. The TLR6 was highly co-expressed with phagosome marker LC3 in the ZNPs-treated macrophages compared with control (Fig. 7a).

Effect of TLR6 on autophagy induced by ZNPs

Next, we examined whether TLR6 had its impact on autophagy. In the TLR6–siRNA + ZNPs group, LC3-II protein was suppressed compared with the ZNPs alone group (Fig. 7b). Taken together, these results clearly showed that TLR6 affects ZNPs-induced autophagy in macrophages.

Influence of TLR6 on phagocytosis

Here, we examined the effect of TLR6 on the ZNPs-induced phagocytosis. Phagocytic activity or internalization of latex beads was decreased in TLR6–siRNA + ZNPs-trea-

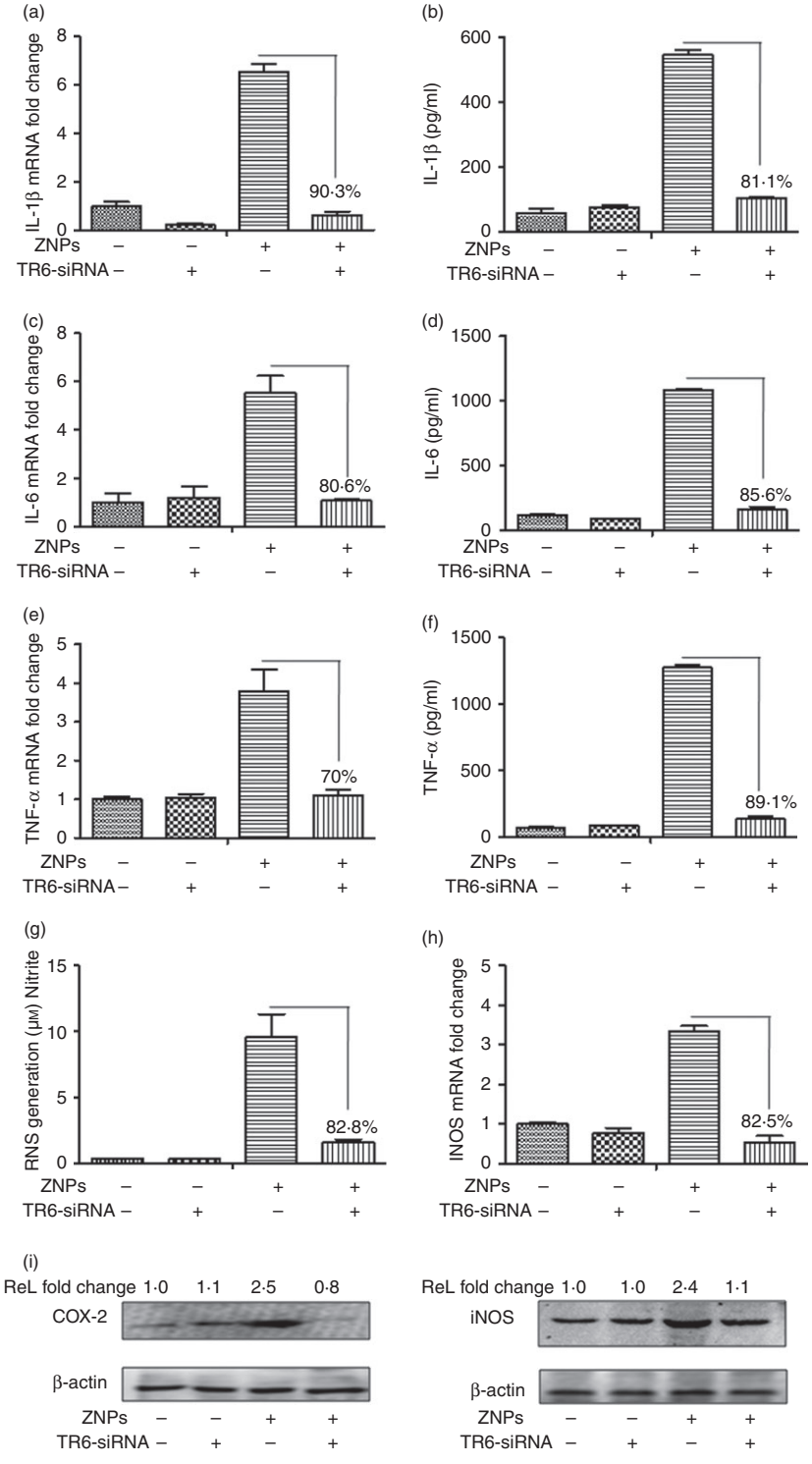

Figure 5. Role of toll-like receptor 6 (TLR6) in zinc oxide nanoparticle (ZNPs) -induced cytokines, inducible nitric oxide synthase (iNOS), cyclo-oxygenase 2 (COX-2) and reactive nitrogen species (RNS). Messenger RNA and protein expression of (a, b) interleukin-1β (IL-1β), (c, d) IL-6 and (e, f) tumour necrosis factor-α (TNF-α) upon TLR6–small interfering (si) RNA transfection followed by ZNPs exposure for 24 hr in macrophages. Data represents mean $\pm$ SE, (n = 3). Effect of TLR6–siRNA transfection on ZNPs-induced (g) RNS generation; (h) iNOS mRNA (data represent mean $\pm$ SE, n = 3 and (i) COX-2 and iNOS protein (relative fold change in protein levels compared with control was denoted above the blots). Blots were reprobed with anti-β-actin to ensure equal loading.

ted macrophages compared with those treated with ZNPs alone (Fig. 7c). This result showed the direct effect of TLR6 on the primary functional property of macrophages.

Discussion

Macrophages are professional phagocytes, responsible for the recognition and engulfment of pathogens. Cell maturation, activation and antigen presentation ability can be estimated by studying the changes of these molecules over the APCs. The study demonstrated that exposure to ZNPs dramatically enhanced the cell activation (CD11b), maturation marker (CD1d) and co-stimulation molecule (MHC-II). These are crucial markers required for macrophages to become highly matured and activated.

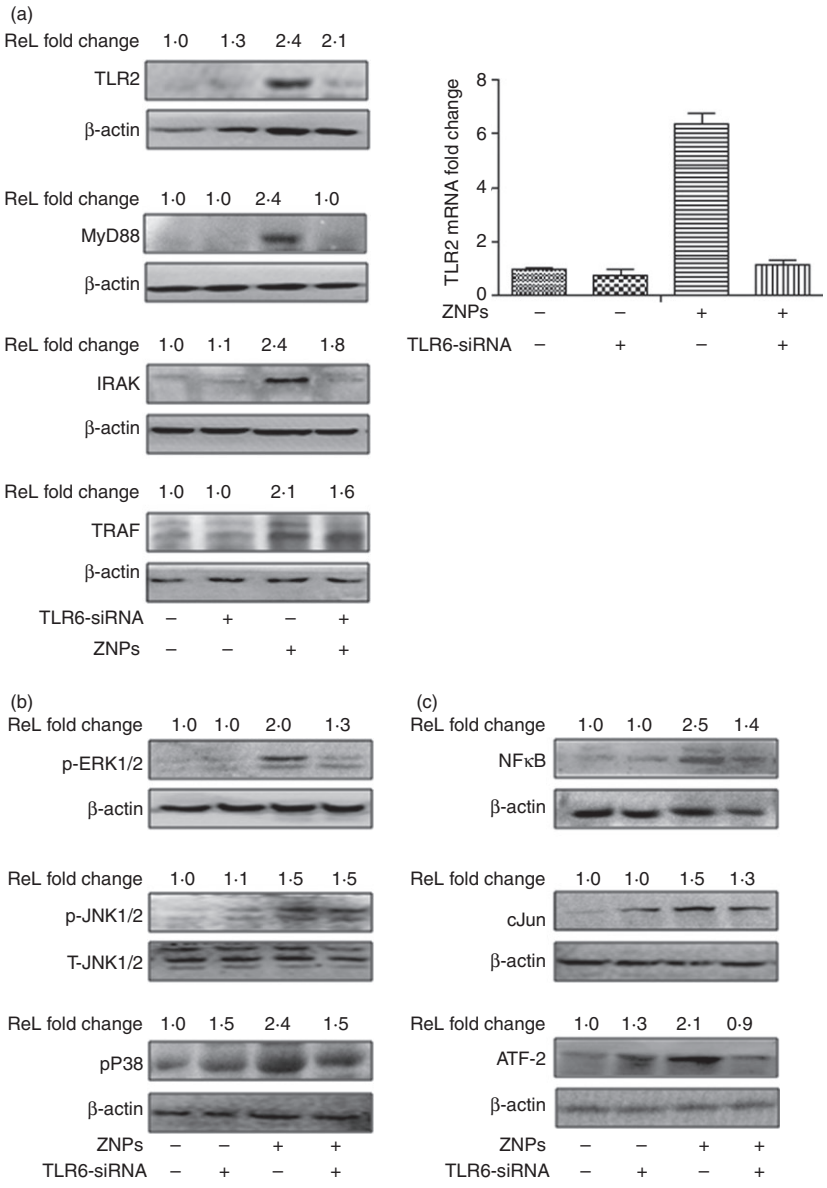

Figure 6. Effect of Toll-like receptor 6 (TLR6) on TLR2, mitogen-activated protein kinases (MAPKs) and transcription factors. Representative immunoblots in TLR6-silenced macrophages (a) TLR2, myeloid differentiation 88 (MyD88), IL-1 receptor associated kinase (IRAK) and tumour necrosis factor receptor-associated factor (TRAF); (b) MAPKs [extracellular signal-regulated kinase (p-ERK1/2), c-Jun-terminal kinase (p-JNK1/2) and phospho-p38] and (c) transcription factors (nuclear factor-κB, cJun and activating transcription factor 2) followed by 2·5 μg/ml zinc oxide nanoparticles (ZNPs) exposure for 24 hr in macrophages. Blots were reprobed with anti-*β*-actin to ensure equal loading and relative fold change in protein levels compared with control was denoted above the blots.

This result was supported by the finding in which exposure of APCs to charged nanoparticles or carbon nanotubes *in vitro* and *in vivo* situations caused an increase in phagocytosis and enhanced the surface expression of MHC-II.[21,22] Recognition of ZNPs induced the expression of cytokines essential for the adaptive immune response as well as required for antigen presentation, such as MHC-II, CD80 and CD86. It has been reported that recognition through TLRs activated these markers on APCs.[23]

Our previous study showed that ZNPs exposure resulted in immunomodulation through the induction of inflammatory responses such as release of pro-inflammatory cytokines as well as activation of MAPKs in macrophages.[17] It is known that ZNPs are potent inducers of inflammatory cytokines and MAPKs, though it is not clear which receptor(s) are responsible for this induction. Therefore, the present study specifically addressed the role of TLRs in the release of inflammatory cytokines that is associated with ZNPs exposure in macrophages. Screening of TLR1 to TLR9 showed the following: transcript levels of TLR1, TLR2, TLR4 and TLR6 were evidently enhanced, while expression of TLR3, TLR5, TLR7, TLR8 and TLR9 were decreased. These results indicated that the size or physicochemical properties of ZNPs may not be enough to activate TLR3, TLR7 and TLR9. Over-expression of TLR2 and TLR6 suggested that ZNPs may interact or form complexes with lipoproteins and peptidoglycans. It is known that TLR2 is involved in the responses to lipoproteins/lipopeptides.[24] Significant expression of TLR6 and TLR2 has been found, which is obvious because when the cytoplasmic domain of TLR2 forms functional

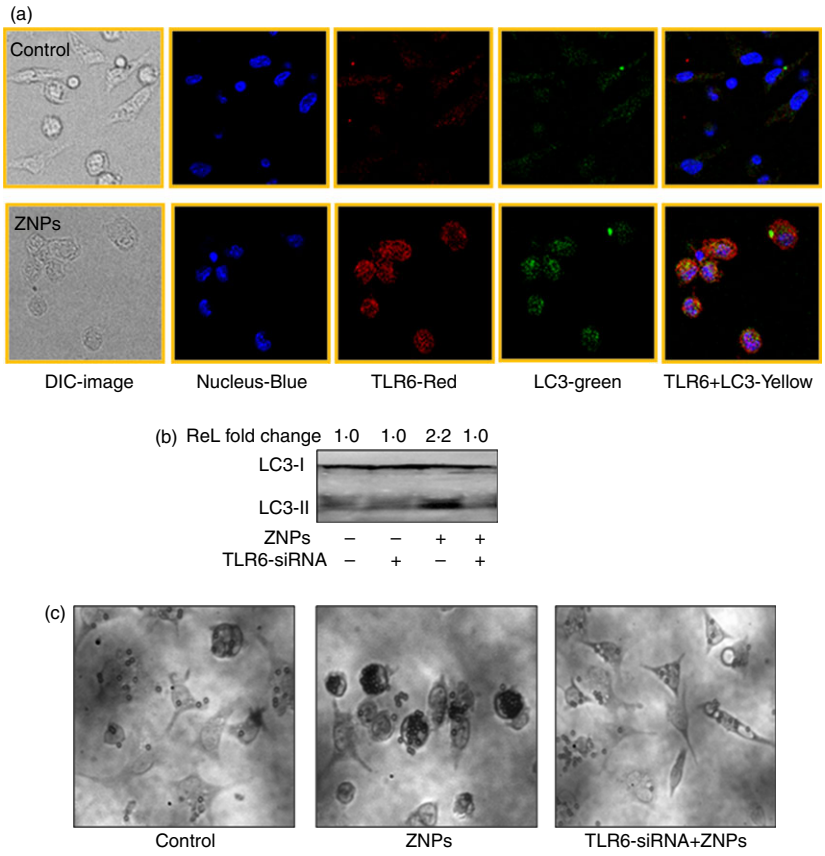

Figure 7. Localization of Toll-like receptor 6 (TLR6) in autophagosomes and its silencing effect on autophagy marker protein and phagocytosis. (a) Localization of TLR6 in autophagosomes (blue: nucleus; green: autophagosomes; red: TLR6). TLR6 silencing effect on the (b) formation of LC3-II and (c) phagocytosis followed by 2·5 μg/ml zinc oxide nanoparticles (ZNPs) exposure for 24 hr in TLR6-silenced macrophages.

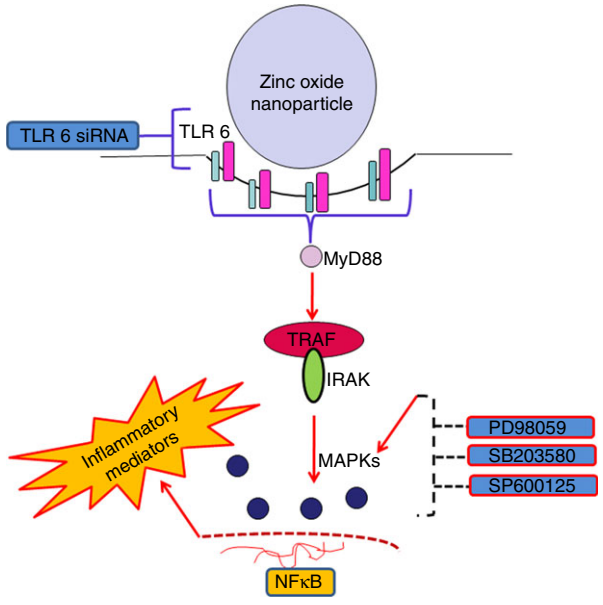

Figure 8. Role of TLR6 and MAPKs signaling on ZNPs induced inflammatory responses.

pairs with TLR6 this leads to cytokine production.[25] Between TLR2 and TLR6, TLR6 was selected for this study because protein expression analysis of TLR2 and TLR6 showed much higher expression of TLR6 than of TLR2 on ZNPs exposure. Ho *et al.*,[20] showed that MyD88, the adaptor protein for most TLRs, was necessary for nanoparticle-induced inflammatory responses. The role of TLR signalling was validated by the activation of its downstream signalling molecules such as MyD88, IRAK and TRAF.

It has been previously shown that inhaled ultrafine ZNPs cause a flu-like illness called fume fever that is accompanied by increased production of IL-1, TNF-α and IL-6.[26–28] Activation of TLRs results in the activation of MAPKs, leading to the expression of pro-inflammatory cytokines.[29] Our previous results showed that ZNPs exposure to macrophages caused activation of MAPKs in the internalization process of ZNPs through endocytosis and during endosome formation ERK1/2 and p38 were found to be activated.[17,30–33] Hence, this study further aimed to address if there was a common link in regulation of inflammatory responses and MAPKs signalling pathways. Our results confirmed the influence of ERK and p38 MAPKs on IL-1β, IL-6 secretion and iNOS expression. This was supported by the finding that IL-1 and IL-6 secretion involve p38 and ERK1/2-MAPKs.[34,35] Reactive nitrogen species and TNF-α are key mediators of the host response to infection. ZNPs-induced release of TNF-α expression was influenced by ERK and p38-MAPKs pathways while the expression of iNOS involves all the MAPKs. Our results were well supported by Ajizian and English,[36] who demonstrated that

ERK and p38 MAPKs are required for iNOS and TNF-α production in murine macrophages.

Involvement of MAPKs enhances inflammatory responses that are directly linked to the macrophage activation by TLRs stimulation.[20,37,38] In an effort to better understand the dependency of MAPKs on TLR6, further experiments were designed by silencing TLR6 in macrophages. TLR6-silenced macrophages showed suppressed inflammatory responses, i.e. decreased p-ERK, p-p38 MAPKs, downstream transcription factors (nuclear factor-κB and ATF2), inhibited TLR2 and downstream TLR signalling (MyD88, IRAK and TRAF). Inhibition of TLR2 in TLR6-silenced macrophages showed the key role of TLR6. Taken together, these results highlighted the major role of TLR6 in recognizing ZNPs and affecting macrophage function. TLR6 was found to be co-localized with autophagosomes in ZNPs-treated macrophages, which emphasizes the direct effect of TLR6 on phagocytosis or the internalization process. According to our hypothesis, the lack of TLR6 had a major effect on the phagocytic activity of macrophages and LC3-II protein formation, which are required for autophagosome formation.

Overall, this study provides insight into the influence of the TLR6 receptor on ERK and p38 signalling and functional responses of macrophages during ZNPs exposure. Indeed, inflammatory stimuli generated by the ZNPs exposure activate macrophages to up-regulate such inflammatory states; therefore TLR6 may be a useful target in the development of new anti-inflammatory drugs for ZNPs-related health problems.

Conclusion

This study suggests that each new nanomaterial should be individually tested before their use in final products. It is clear from this study that the induction of inflammatory responses after exposure of ZNPs is directly related to TLR6. The ZNPs activated macrophages by enhancement of CD11b, CD1d and MHC-II to initiate immune responses that are essential for functional properties of macrophages. The generation of these responses depends on TLR6-mediated ERK and p38 pathways in macrophages (Fig. 8). This study will contribute towards better understanding of the mechanisms underlying the pathogenesis of ZNPs-induced damage leading to fever or illness and point towards TLR6 as a potential target for the development of novel anti-inflammatory therapeutics.

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Disclosures

The authors declare no competing financial interest.

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